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⑯ Applicant: MASSACHUSETTS INSTITUTE OF TECHNOLOGY, 77 Massachusetts Avenue, Cambridge, MA 02139 (US)

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⑯ Inventor: Rha, ChoKyun, 285 Commonwealth Ave, Boston Massachusetts 02115 (US)  
Inventor: Rodriguez-Sanchez, Dolores, 60 Wadsworth St., 3c, Cambridge Massachusetts 02142 (US)

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⑯ Representative: Patentanwälte Zellentin,  
Zweibrückenstrasse 15, D-8000 München 2 (DE)

⑯ Process for encapsulation and encapsulated active material system.

⑯ Capsules are formed having a liquified core while avoiding capsule core gelatin by adding drops of a solution of either an anionic polymer composition or a cationic polymer composition to a solution of an ionic polymer of opposite charge. The drops contain an active ingredient such as a cell or microorganism capable of producing a biologically active product or can contain a biological or chemical composition. The interface of the ionic polymers form a permeable membrane surrounding the liquid drops.

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5 Process for Encapsulation and Encapsulated Active  
Material System

This invention relates to a process for encapsulating  
biologically active materials such as cells or tissues  
10 or biochemically or chemically active compositions and  
to the encapsulated system including the active materials.

In biochemical production and biotechnological applica-  
tions, health and viability of active materials such as  
15 cells, microorganisms and the like, is important since  
these active materials are capable of producing  
biologically or biochemically active components that find  
a wide variety of use. For example, cells are capable  
of producing antibodies, hormones, lymphokines, anti-  
20 biotics, interferons and other biochemicals or chemicals.  
Mammalian cell lines are grown by being surrounded by  
an aqueous medium containing a nutrient in order to  
promote the viability and growth of the cells and enables  
continued production of the desired microbiological or  
25 biological products. It has been proposed to utilize  
so-called microcarriers, which are beads having the  
appropriate charge and exchange capacity to promote  
the growth of the cells thereon in an efficient manner.  
The microcarriers themselves are maintained in an  
30 aqueous suspension containing the proper nutrient  
composition to promote cell growth and production of  
the desired microbiological product.

35 Biological products which are shed or excreted from the  
cells become admixed with the aqueous suspending

1 composition, which in many cases, is at very dilute concentrations. The subsequent recovery of the desired product is thereby rendered difficult and time consuming.

5 In order to overcome problems associated with micro-  
biological product recovery, it has been proposed to  
encapsulate cells or microorganisms within a membrane  
which permits nutrients to be metabolized by the cell  
or microorganism while retaining the microbiological  
10 product produced by the cell or microorganism within  
the encapsulating membrane. Such processes are disclosed,  
for example, in U.S. Patents 4,409,331 and 4,352,883.  
The semipermeable membrane surrounding the biologically-  
active material has a selected permeability so that  
15 substances having a certain molecular weight or below,  
are allowed to pass through the semi-permeable membrane.  
By controlling the permeability of the membrane, and  
by having a knowledge of the approximate molecular size  
of the desired product, one can confine the product,  
20 within the space between the active material and the  
semi-permeable membrane.

Unfortunately, the process described in U.S. Patent  
4,409,331 and 4,352,883 require that the membrane be  
25 formed from the surface of an initially formed solid  
gel bead. This requires that the interior of the bead  
be subsequently liquefied so that the diffusion of  
nutrient which is required by the microorganism or  
cell, will not be hindered thereby to promote formation  
30 of the desired microbiological product. Furthermore,  
liquefaction of the gel is highly desired so that the  
space between the semi-permeable membrane and the  
microorganism or cell is available for either cell  
production or products. Typically, these prior art  
35 membranes are formed from a cell suspension in alginate

1 solution which is added dropwise to a calcium chloride  
aqueous solution, thereby to form solid gel beads. The  
beads then are washed with N-cyclohexylamine ethane  
sulfonic acid (CHES) and then washed subsequently with  
5 sodium chloride. Thereafter, a polylysine solution is  
added to form a polymer complex with the alginate  
surface. This surface then is washed with CHES/sodium  
chloride, subsequently with calcium chloride and then  
subsequently with sodium chloride. The membrane then  
10 is incubated and the gel within the membrane is  
subsequently liquefied by washing twice with sodium  
chloride, incubating, washing with sodium citrate and  
sodium chloride, washing with sodium chloride, and then  
a final wash. Obviously, such a process for forming  
15 encapsulated microbiologically active ingredients is  
time consuming and difficult and requires a high level  
of laboratory technique in order to successfully  
produce the encapsulated cell or microorganism suspended  
in a liquid medium. Furthermore, during these complicated,  
20 time-consuming steps, the viability, productivity or  
other characteristics of the cell may be altered.

It would be highly desirable to provide a means for  
25 encapsulating a microorganism or cell capable of  
producing a biologically active material which eliminates  
the necessity of liquefying a solid carrier in order  
to promote mass transfer into and out of the cell  
or microorganism. Furthermore, it would be desirable  
30 to provide such an encapsulating means which is capable  
of drastically reducing the number of steps needed to  
form the encapsulated cell or microorganism. In  
addition, it would be desirable to provide such an  
encapsulating means which permits the formation of  
35 a membrane capable of having a permeability over  
a wide range, which permits the isolation or selective

1 separation of a wide variety of biologically or chemically  
active molecules.

5 In accordance with this invention, cells, microorganisms,  
or the like, capable of producing a biologically active  
composition or biochemicals such as enzymes or hormones  
or the like or nonbiochemical compositions such as  
substrates, reactants, or catalysts are encapsulated by  
a polymer complex comprising the combination of an anionic  
10 polymer and a cationic polymer. The term "active mater-  
ial" is used herein to include cells, microorganisms or  
the like which produce a biologically active composition  
or a composition such as an enzyme, hormone, antibody,  
antibiotic insecticide, catalyst, substrate or reactant  
15 or the like which active material is to be encapsulated  
in accordance with this invention. The active material  
is suspended in an aqueous solution of either one of the  
cationic polymer or the anionic polymer composition.  
The polymer composition containing the active material  
20 then is formed into non gel liquid particles and is  
added to the other polymer such as in the form of drops  
from a capillary tube or a spray or the like to form  
capsules comprising a membrane surrounding a liquid core.  
The active material is housed within the interior of the  
25 membrane suspended in the liquid core. The capsules then  
are washed and ready to use or then can be stored in an  
appropriate medium until use. The permeability of the  
membrane is controlled by controlling concentration of  
the cationic and anionic polymers in the solution used  
30 in the preparation of the capsule, the pH of the aqueous  
solutions in which the cationic polymer or anionic  
polymer are prepared, the presence or absence of counter-  
ions in each solution, and the molecular weight of the  
anionic polymer and the cationic polymer as well as  
35 the selection of specific polymers.

1 The process of this invention eliminates the need for  
liquefying the core of the capsule containing the active  
ingredient and also eliminates the need for multiple  
washing steps with a variety of reagents which may  
5 adversely affect the biological, biochemical or chemical  
activity of the active ingredient to be encapsulated.  
In addition, the process of this invention is useful  
with a wide variety of biologically active molecules  
over a wide molecular type and weight range, since the  
10 permeability of the membrane formed around the capsule  
can be varied widely. Thus, it is possible to separate,  
isolate or selectively segregate biologically active  
compounds of varying nature by controlling the perme-  
ability of the membrane.

15 In accordance with this invention, an active material  
comprising or being capable of producing biologically  
active compositions is encapsulated within a membrane  
capable of permitting transport of a variety of compounds  
20 such as a nutrient for a cell to the active material  
and capable of selectively containing, within the mem-  
brane, the compound produced. The active ingredient can  
be a cell, microorganism, tissues or chemical or bio-  
chemical reactants. Representative suitable cells  
25 include fused cells, eg. hybridoma cells, or genetically  
modified cells produced by recombinant DNA technology  
and lymphocyte cells capable of producing antibodies  
or microorganisms for fermentation.

30 In addition, microorganisms such as bacteria, can be  
encapsulated in accordance with this invention.  
Furthermore, biologically active compositions such as  
35 antibiotics, enzymes, hormones, <sup>antibodies</sup> or the like can be  
encapsulated so that they can be controllably released  
through the membrane or retained therein if desired.

1 The encapsulated active ingredient is enclosed by the membrane, which also can contain an aqueous medium which includes nutrients for the active ingredient. The aqueous medium also is capable of dissolving or sus-  
5 pending the microbiologically active material produced by the active ingredient without degrading it. The perme-  
ability of the membrane is such as to permit passage of nutrients from a medium surrounding the membrane into the aqueous medium enclosed by the membrane, and so that  
10 the microbiologically active composition can be produced by the active ingredient.

15 The active ingredient first is suspended in an aqueous solution of either (a) one or more anionic polymers or (b) one or more cationic polymers. The anionic polymers or cationic polymers chosen are formed of molecularly  
repedative segments linked together which here are either positively charged or negatively charged segments distributed along the chain or on substitutions distribut-  
20 ed along the chain. The concentration of charged segments is such as to permit electrostatic interaction and en-  
tanglement of the polymers when they are contacted together thereby to form the membrane. The resultant suspension then is sprayed into or added dropwise or the  
25 like as liquid particles to the other polymer so that a membrane is formed at the interface between the anionic polymer and the cationic polymer. When the interface between the two oppositely charged polymers encloses the active ingredient, the active ingredient thereby becomes encapsulated. Representative suitable  
30 anionic polymer include alginate, carragenen, hyaluronic acids, carboxymethylcellulose, xanthan, furcellaran and, sulfonated organic polymers, usually in salt form, eg, sodium salt. Representative suitable  
35 cationic polymers include chitosan, polylysine,

1 polyethylamine and polyvinylamines as well as other  
2 amine or imine containing polymer which is capable of  
3 coacting with an anionic polymer to form a membrane. The  
4 preferred anionic polymers are alginate, or carragenan.  
5 The preferred cationic polymers are chitosan, or poly-  
6 lysine. The droplets of the charged polymer containing  
7 the active ingredient can be regulated in order to  
8 regulate the size of the final encapsulated product.  
9 Typical encapsulated products have a size within the  
10 range of about 50 microns and 5000 microns. When cells  
11 are to be encapsulated, the capsule has a size which  
12 permits oxygen transfer to those cells that require  
13 oxygen for vitality and has a size sufficiently small  
14 to afford efficient isolation of the desired cell product,  
15 typically between about 400 and 800 microns.

20 The permeability of the membrane formed by the inter-  
21 action of the anionic polymer and the cationic polymer  
22 is controlled by controlling the relative concentration  
23 of the two oppositely charged polymers, their concentrat-  
24 ion in the individual aqueous media, the pH of each of  
25 the polymer solutions, the molecular weights of the  
26 polymers and presence or absence of counter-ions in  
27 either of the solutions. By the term "counter-ions" is  
28 meant ions which interact with the charged portion of  
29 the polymer in order to reduce interaction of that  
30 polymer with the oppositely charged polymer. For  
31 example, calcium ion interacts with carboxyl ion on  
32 the anionic polymer. The calcium ion can be removed  
33 with phosphate ion. Increased polymer concentration  
34 usually results in decreased permeability. A decrease  
35 in the pH of the anionic polymer composition results  
in increased concentration of hydrogen ion thereby to  
form reactive cations on the cationic polymers having  
amine or imine groups. The achievement of a membrane

1 having a desired permeability can be determined by  
varying the process parameters and incorporating a  
mixture of compounds of anions molecular weight and size  
in the droplets or spray. The aqueous medium outside the  
5 capsules thus produced can be assigned for the presence  
of these compounds so that the molecular weight/molecular  
size cut-off level of the membrane is thus determined.

This invention also provides capsules having a normal  
10 membrane structure having improved mechanical properties  
as compared to the capsules of the prior art. Membranes  
produced from a gel composition and which are subsequently  
liquefied have reduced strength. This is due primarily  
15 to the fact that a large proportion of the polymer chains  
becomes oriented towards the interior of the capsule  
during gelation rather than in the plane of the membrane.  
During liquification of the gel, these portions of the  
polymer chain do not become reoriented into the plane  
20 of the membrane and therefore do not contribute to  
membrane strength. In contrast, in this invention, the  
ionic portions of the anionic and cationic polymers need  
not be encumbered with counter ions so that they are  
free to react with each other along the entire chain  
25 length where the different polymers come into reactive  
contact. By operating in this manner, larger chain  
lengths of the polymers are oriented in the plane of  
the membrane. In one particular aspect of this invention,  
it is possible to have the anionic polymer oriented  
30 on the outside of the membrane rather than on the inside  
of the membrane. Thus, for example, alginate can  
comprise the outer membrane surface. The result is not  
possible with prior art processes since the alginate  
is required to form the initial gel bead. Thus, this  
35 invention provides the user with much greater flexibility  
in forming the capsule. In another particular

1 aspect of this invention, multi-membrane walls can be  
formed thereby providing membranes with greater strength  
as compared to capsule of the prior art. This is  
accomplished by forming the capsule with the anionic  
5 polymer chain on the outside of the membrane by the  
process set forth above. The capsules then are separated  
from the surrounding aqueous medium by any convenient  
method such as filtration or centrifugation. The capsules  
then are mixed with a solution of anionic polymer and  
10 a crosslinking divalent metal ion. For example, in the  
case of alginate as the anionic polymer, calcium ion or  
barium ion can be used as the crosslinking divalent ion  
to form an outer membrane of alginate polymer.

15 After the encapsulated active ingredients are produced  
in accordance with the above-described process, then  
they can be separated from the aqueous medium where  
they are suspended, and then reintroduced into an aqueous  
medium which contains the nutrients for the active  
20 ingredient, so that the microbiologically active  
compound can be produced. On the other hand, the  
nutrients can be added to the suspension of encapsulated  
active ingredients without prior separation thereof.

25 The following examples illustrate the present invention  
and are not intended to limit the same.

Example I

30 An alginate solution comprises 0.75 percent--1 percent  
w/v sodium alginate and 150 mM NaCl was added dropwise  
to a chitosan solution. The chitosan solution comprised  
0.05--0.10 gr/dl chitosan, 117 mM NaCl, 0.01 M CaCl<sub>2</sub>  
and 0.01 M HCl. The chitosan solution had a pH of  
35 6.5. The alginate solution was added dropwise to the  
chitosan solution to form capsules which were incubated

1 in the chitosan solution for about one minute. Samples  
2 of the chitosan solution containing the capsules were  
3 separated by centrifugation or by filtration on a  
4 centered glass filter, washed with water and transferred  
5 separately to a phosphate buffer solution, a saline  
6 solution, distilled water or a cell culture medium  
7 comprising Dulbecco's Modified Minimum Essential  
8 Medium, 5 % Fetal Calf Serum and 5 % Calf Serum and were  
9 found to be surprisingly stable. In addition, the cap-  
10 capsules were found to be able to sustain centrifugation  
11 at a level at least as high as about 2000 RPM for 10  
12 minutes. In this example, it is preferred that the  
13 alginate solution have a viscosity higher than about  
14 3.0 centistokes while a chitosan solution preferable  
15 has a viscosity of at least about 1.5 centistokes.

Example II

20 Following the procedure of Example I, capsules were  
21 formed by adding a chitosan solution dropwise to an  
22 alginate solution. The chitosan solution comprised  
23 1.5 percent w/v chitosan, 2.5 percent citric acid and  
24 0.07 M  $\text{CaCl}_2$ . The alginate solution comprised either  
25 1.1 percent w/v sodium alginate and 0.5 percent sodium  
26 sulfat, or a solution comprising 1 percent w/v sodium  
27 alginate. As in Example I, the capsules were found to  
28 be stable in phosphate buffer, saline, water and cell  
29 culture medium, and were able to sustain centrifugation  
30 at a level of about 2000 RPM for at least 10 minutes.  
The core of the capsules is rendered more fluid-like  
31 and less solid-like by lowering the concentration of  
32 calcium chloride in the chitosan solution.

Example III

35 Following the procedure of Example I, capsules were  
36 formed by adding chitosan solution dropwise in an

1 alginic solution to obtain capsules with a liquid core. The chitosan solution utilized contained between 0.1 percent and 1.5 percent w/v chitosan, 0.05 M NaCl and between 0.006 M and 0.2 M  $\text{CaCl}_2$  and a pH rate ranging 5 between 5.5 and 6.6. The alginic solution ranged between 0.1 percent and 1.0 percent sodium alginic.

As in Examples I and II, the capsules produced were found to be stable in phosphate buffer, saline, water and in 10 the cell culture medium. As shown in Table I, the rupture strength of the capsules produced by this invention can be increased by treating them with a divalent ion after they are formed. Alternatively, the divalent ion can be added with an ionic polymer with 15 which it does not interact to form a gel. The diffusion properties can also be controlled by solution conditions which influence the molecular configuration or the charge density of the polymers. The rupture strength of various capsules made in accordance with the 20 procedures set forth in Example III is shown in Table I.

Table I Effect of divalent cations on the rupture strength of the capsules

Cation	Capsule Preparation	Rupture Strength g/cm <sup>2</sup>
$\text{Ca}^{+2}$	1.35 % chitosan, 0.05 N in 0.5 % alginic; no treatment of capsules	8
$\text{Ca}^{+2}$	Prepared as above + treatment of capsules in 0.1 M $\text{CaCl}_2$ for 30 5 minutes	743
$\text{Ba}^{+2}$	1.35 % chitosan, 0.05 M $\text{BaCl}_2$ dropped in 0.5 % alginic; no further treatment	696
$\text{Ba}^{+2}$	Prepared as above + treatment of capsules in 0.1 M $\text{BaCl}_2$ for 35 5 minutes	1609

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Massachusetts Institute  
of Technology  
Cambridge / MA 02139

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10 U.S.A.

15 Claims

1. A process for producing a capsule having a liquid core from ionic polymers while avoiding gelation of said core which comprises:  
20 Forming a liquid droplet from an aqueous solution of a first ionic polymer selected from the group consisting of at least one anionic polymer and at least one cationic polymer and by contacting said droplet with a  
25 solution of at least one second ionic polymer, said second ionic polymer having an ionic charge opposite from said first ionic polymer and reacting said first and second polymer in contact with each other thereby to form a membrane encapsulating said droplet.
- 30 2. A process of claim 1 wherein said droplet contains an active material wherein said active material may be one of the group consisting of living cells or microorganisms or hybridoma cells or lymphocytes

1 or bacteria or a biologically active compound or an enzyme or a hormone.

5 3. A process for producing a product from a living cell which comprises encapsulating said cell by the process of claim 1 and controlling the permeability of said membrane to prevent said product from permeating said membrane, collecting said encapsulated cell, rupturing said membrane and recovering said product, wherein said cell may be one of the group consisting of a hybridoma or a lymphocyte cell.

10 4. A process of claim 1, 2 or 3, wherein said anionic polymer is selected from the group consisting of alginate and carragenan and said cationic polymer is selected from the group consisting of chitosan and polylysine.

15 20 5. A process of anyone of claims 1 to 4, wherein a second membrane is formed by cross-linking a second anionic polymer to the anionic polymer portion of said first polymer by adding a divalent metal cation and said second anionic polymer after said first membrane is formed, said metal cation may be selected from the group consisting of calcium, barium and mixtures thereof.

25 30 6. A capsule comprising a polymeric membrane surrounding a liquid core wherein said membrane is formed by the interaction of at least one anionic polymer with at least one cationic polymer and wherein molecular chains comprising said polymer are oriented substantially whithin said membrane.

- 1 7. The capsule of claim 6, wherein said cationic polymer forms the inner surface of said membrane adjacent liquid core.
- 5 8. The capsule of claim 6, wherein said anionic polymer forms the inner surface of said membrane adjacent said liquid core.
- 10 9. The capsule of claim 6, wherein said liquid core contains an active material, wherein said active material may be one of the group consisting of living cells, hybridoma cells and lymphocyte cells.
- 15 10. The capsule of anyone of claims 6 to 9, wherein said anionic polymer is selected from the group consisting of alginate and carragenan or wherein said cationic polymer is selected from the group consisting of chitosan and polylysine.
- 20 11. The capsule of anyone of claims 6 to 10, which includes a second membrane formed by cross-linking the anionic polymer to the anionic polymer portion of said polymeric membrane with a divalent metal ion, wherein said divalent metal ion may be selected from the group consisting of calcium, barium and mixtures thereof.
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